



Cellular Senescence in Liver Disease and Regeneration

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Abstract

Cellular senescence is an irreversible cell cycle arrest implemented by the cell as a result of stressful insults. Characterized by phenotypic alterations, including secretome changes and genomic instability, senescence is capable of exerting both detrimental and beneficial processes. Accumulating evidence has shown that cellular senescence plays a relevant role in the occurrence and development of liver disease, as a mechanism to contain damage and promote regeneration, but also characterizing the onset and correlating with the extent of damage. The evidence of senescent mechanisms acting on the cell populations of the liver will be described including the role of markers to detect cellular senescence. Overall, this review intends to summarize the role of senescence in liver homeostasis, injury, disease, and regeneration.

Keywords

- senescence
- liver disease
- liver regeneration

We are living longer than at any point time in human history.¹ Life expectancy has increased in the developed world from an average 40.2 years in 1891 to 82.8 years in 2018 (for females in United Kingdom²).

At the beginning of the 20th century, the epidemiological transition that led to more than a doubling in the average lifespan sparked the idea that ever-increasing age and near immortality could be reached if the human being was provided with appropriate nutrition and correct health interventions (e.g., child immunization).³

It was in this context, in an era of rapid change, when the controversial Nobel Prize laureate and eugenicist Alexis Carrel propelled this idea of immortality.⁴ In his experiments, Carrel, who was attempting to preserve life outside the body, claimed to have maintained cells from chick heart embryos alive and dividing from 1912 to 1946.^{5,6} The implicit idea of these studies was that by understanding cellular mechanisms, the secrets of an organism's immortality could be revealed.⁷

This concept was challenged when Hayflick and Moorhead described that human cells in culture showed a limited proliferative capacity and entered cellular senescence.⁸ Hayflick and Moorhead observed how the cells adapted to the culture conditions and began to proliferate but, after certain number of divisions, they invariably changed their morphology, remaining metabolically active but unable to divide again (despite the presence of growth factors in the medium).⁸

Hayflick termed this phenomenon *cellular senescence*, and despite the initial reluctance of his colleagues to accept that primary cell cultures were mortal,⁶ subsequent publications helped define a new phenotype, the function of which continues to be unraveled.

Cellular Senescence in Homeostasis and Disease

Our cells and tissues are constantly exposed to injury, including the liver, subjected to injury due to its role in toxin metabolism and susceptibility to infections.⁹

In response to a variety of stresses, cells either undergo apoptosis (a form of programed death) or senescence.¹⁰ Although each mechanism has its own advantages, in a context where a cell becomes mutated, senescence protects the integrity of the tissue by preventing proliferation of these cells and the transmission of destructive alterations to the next generation that may give rise to further injury or cancer.¹¹

Senescence is therefore considered a preventive mechanism against tumor formation. This is supported by the fact that tumorigenesis selects cells that can bypass senescence^{12,13} and that alterations of key effectors of senescence, such as p53, are common in tumors.¹⁴

When one of these cells acquires a senescent phenotype, it is no longer able to proliferate, but remains metabolically

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active and is able to communicate with the surrounding environment.¹¹ To do so, senescent cells secrete a myriad of factors that act in an autocrine and paracrine manner to reinforce senescence, communicate its compromised status to the rest of the tissue, and to activate immune surveillance to promote clearance of the damaged area and induce regeneration.¹⁵ It is therefore an essential mechanism activated during tissue damage, wound healing,¹⁶ tissue plasticity, and regeneration.^{17,18}

In line with this protective role, senescence can be activated in postmitotic nonmalignant cells that if uncontrolled could aggravate certain pathologies. For example, cellular senescence of scar-forming cells attenuates fibrosis in the liver,^{19,20} kidney,²¹ heart,²² and skin,^{16,23} restraining uncontrolled scarring and providing a regenerative environment.

Senescence is also part of a normal physiological response in adults. Normal megakaryocytes,²⁴ natural killer (NK) cells at the interface between fetus and mother²⁵ and the syncytiotrophoblast at the placenta,²⁶ undergo senescence as part of their normal maturation. Senescence is essential for the correct functioning of these systems and might provide protective mechanisms later in life.²⁶

Senescence has also been described to fine-tune embryo development, promoting a correct balance between cell populations during embryo patterning as well as eliminating transient developmental structures.^{27,28}

As an antitumoral, proregenerative, homeostatic, and developmental mechanism, senescence shows its positive physiological sides. However, senescence is a highly complex and plastic phenotype that can exert antagonistic effects depending on the context.

For example, senescent cells contribute to persistent chronic inflammation (“inflammaging”)^{29,30} as well as drive tumorigenesis by promoting angiogenesis and cell motility (i.e., invasion, migration, and metastasis).³¹ Furthermore, chronic senescence and a maintained senescence-associated secretory phenotype (SASP) have been shown to actively contribute to several pathological conditions such as atherosclerosis,³² diabetes,³³ osteoarthritis,³⁴ obesity,³⁵ and neurodegenerative diseases.³⁶ The list of conditions in which senescence negatively contributes to the development of the disease continues to increase, and for many conditions its role still needs to be clarified.

Cellular senescence is also one of the main hallmarks of aging.³⁷ Senescent cells appear to increase in number in a variety of aged mammalian tissues,^{38–42} either due to the accumulation of potentially deleterious mutations or due to the decline in senescence-clearing mechanisms with age.^{30,43} This persistence leads to local and systemic inflammation, procarcinogenic effects, and the emergence of a pernicious microenvironment that promotes further injury.^{15,43}

In summary, senescence is capable of exerting both detrimental and beneficial processes. This seemingly antagonistic pleiotropy might be partially explained by the fact that the interaction of the senescent cells with their microenvironment is highly dependent on the context (i.e., stress inducers, cell type, rapid vs. persistent). In general,

a transient response (in which the senescent cells are rapidly cleared) promotes tissue regeneration, whereas persistent senescence can have devastating effects for the organism.

Features of Cellular Senescence: An Overview

Senescence is a highly heterogeneous response that spatially and temporally depends on the context (inductor, duration of stimulus, cell type, etc.). This heterogeneity explains the lack of a universal biomarker, forcing researchers in the field to identify several traits to asseverate the presence of senescence. Recently, the International Cell Senescence Association proposed a consensus of markers to help define this phenotype including cell-cycle arrest, macromolecular damage, deregulated metabolism, and SASP.¹¹ It is important to understand that not all of these characteristics need to be present, but a combination is required to determine the presence of senescence^{11,15} (► Fig. 1).

The hallmark of senescence is an irreversible *cell-cycle withdrawal* instigated by stressful insults including aberrant proliferation.^{11,43} Two families of cyclin-dependent kinases (CDK) inhibitors are in charge of maintaining this proliferative arrest: INK4 family (comprising p16^{INK4A} [CDKN2A], p15^{INK4B}, p18^{INK4C}, and p19ARF^{INK4D}) and Cip/Kip family (including p27^{Kip1}, p57^{Kip2}, and p21^{WAF1/Cip1} [CDKN1A] which is regulated by p53). These factors inhibit the activity of CDKs by maintaining the retinoblastoma (RB) family members in their hypophosphorylated form, complexed to E2F family transcription factors. RB-dependent repression of E2F activity results in cell-cycle arrest, which cannot be reversed by inactivation of RB or p53.^{44,45} This irreversible arrest is also associated with an active blockage of apoptosis mediated by antiapoptotic BCL-2 family members.⁴⁶

Given that senescence is a result of stressful insults, it is not surprising that DNA damage response (DDR) markers characterize its phenotype. DDR is an evolutionary conserved pathway that senses DNA damage, amplifies its signal, and engages with the cell cycle through senescence effectors.^{47,48} It can be assessed by cumulative activation of the double-strand break sensor proteins γH2A.X, 53BP1, CHK2, and MDC1. When the damage persists, these DDR foci become permanent generating DNA-SCARS, DNA segments with stable chromatin alterations characterized by the presence of single-stranded DNA, and lack of DNA-repair proteins (RPA, RAD51).^{48–51}

Senescence is also associated with chromatin changes including senescence-associated heterochromatin foci (SAHF, facultative heterochromatin domains that contribute to silencing of proproliferative genes^{52,53} visualized as DAPI-dense foci⁵⁴) and senescence-associated distension of satellites (SADS, where centromeres' DNA unraveled from its compact state preceding SAHF's formation⁵⁵). Other common signs of DDR in senescence include oncogene activation/loss of tumor suppressors,⁵⁶ presence of micronuclei/nucleoplasmic bridges or nuclear buds,^{57,58} and loss of Lamin B1 (a component of the nuclear lamina

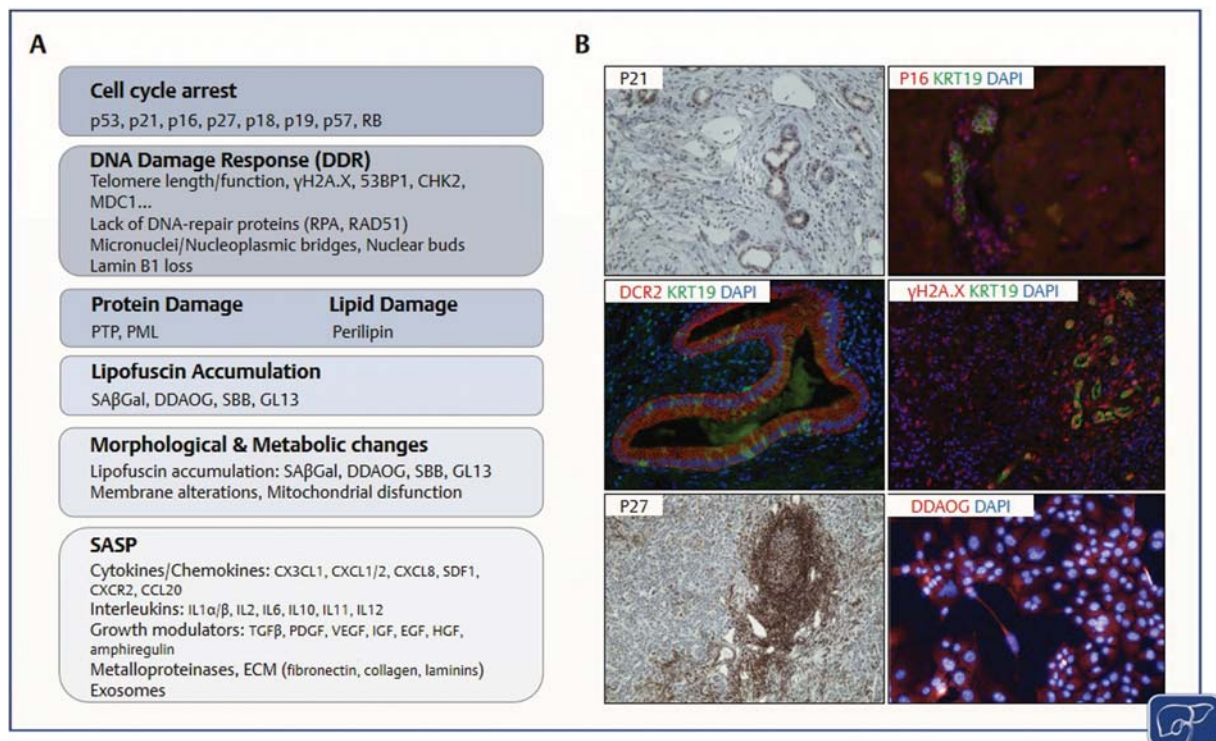


Fig. 1 Hallmarks of senescence in liver disease. (A) Hallmarks of senescence, including cell-cycle arrest,^{11,42} DDR,^{55,56} protein and lipid damage, lipofuscin accumulation,^{37,72–75} morphological and metabolic changes,⁸³ and production of senescence-associated secretory phenotype (SASP).^{15,30,90} (B) Markers of senescence in liver disease. The images represent immunohistochemistry or immunofluorescence of: P21 in primary biliary cholangitis (PBC); P16 (red) in keratin 19-positive cholangiocytes (KRT19, green) in chronic rejection; decoy receptor 2 (DCR2, green) in Krt19-positive cholangiocytes (red) in PBC; γ H2A.X (red) in KRT19-positive cholangiocytes (green) in PBC; P27 in primary sclerosing cholangitis (PSC); DDAOG showing lipofuscin accumulation of mouse cholangiocytes treated with TGF β for 24 hours. All images were taken at 20 or 40 $\times$. DAPI is shown in blue. DDR, DNA damage response.

which triggers both local and global modifications in chromatin methylation and correlate with SAHF and SADS^{59,60}).

Telomere erosion (the progressive shortening of the specialized nucleoprotein structures that protect chromosome ends) has been shown to induce and stabilize senescence while contributing to a persistent DDR response.^{61–65} Therefore, length shortening and presence of telomere-associated foci (TAFs, where markers of DNA damage such as γ H2A.X colocalize with telomeres⁶⁶) are frequently used as senescence biomarkers.

Damage also impacts at the protein level where the lysosomal content increases and aggregates, forming oxidized complexes known as lipofuscin.^{38,67} Lipofuscin accumulation can be detected by using SA β Gal,³⁸ far-red fluorescence probes such as DDAOG,^{68,69} Sudan Black B (SBB) staining⁷⁰, or SBB analogues such as GL13.⁷¹ Other types of protein damage include protein tyrosine phosphatases (PTPs^{72,73}; detected by monoclonal antibodies that recognize oxidized cysteine⁷⁴) and protein carbonyl residues resulting from protein carbonylation by reactive oxygen species (ROS).⁷⁵ Finally, as protein damage is not reversible, activation of the ubiquitin proteasome system^{73,76} and presence of PML (which act as ROS sensors⁷⁷) can also be useful to assess senescence.

Senescent cells also display *morphological changes* becoming larger, flatter, increasing lysosomal content,⁷⁸ and the cytoplasm and nucleus appear vacuolised,^{79,80} although

this is difficult to assess and less obvious *in vivo* due to the architectural support of the tissue. Changes in the plasma membrane are likely representative of the *lipid damage* that occurs in senescent cells (such as lipid-derived aldehyde modifications⁸¹ or lipid accumulation that can be detected using immunostaining for lipid-associated proteins such as perilipin⁸²).

Furthermore, senescent cells show an *abnormal metabolic profile*, and, although they remain metabolically active, display *mitochondrial dysfunction* with more abundant mitochondria, decreased membrane potential, increased mass, and abundance of tricarboxylic acid-cycle metabolites.⁸³ They also have compromised ATP production and produce more ROS,⁸⁴ potentiating the senescence effects.⁸⁵ However, as metabolic changes are a commonality with other cellular processes, it is not a consistent marker of senescence.

Finally, senescent cells secrete a variable set of factors termed the Senescence-Associated Secretory Phenotype (SASP) responsible for the beneficial and deleterious effects of senescence within the tissue.^{15,86–88}

The SASP comprises a highly dynamic network⁸⁹ of cytokines and chemokines (CX3CL1, CXCL1/2, CXCL8, SDF1, CXCR2, CCL20), interleukins (IL1 α/β , IL2, IL6, IL10, IL11, IL12), angiogenic factors and growth modulators (TGF β , PDGF, VEGF, IGF, EGF, HGF, amphiregulin), metalloproteinases (MMP), extracellular matrix (ECM; fibronectin, collagen, laminins), and exosomes.^{90,91} These factors allow the

senescent cell to communicate its compromised state, reinforcing cell-cycle arrest in an autocrine manner (therefore avoiding a potential bypass toward carcinogenesis^{86,87}), spreading senescence to sensitize neighboring cells to the presence of stressful stimulus, and engaging the innate immune system for clearance of senescent cells.^{92–94}

Cellular Senescence in the Liver

The liver's roles in the metabolism of toxins and its susceptibility to infectious agents make it prone to injury. In this context, senescence may be a mechanism to contain damage and promote regeneration. Whether senescence represents a consequence of the damage, an inductor agent, or an intermediate stage of disease remains unclear; however, it is a common factor across etiologies. Here we disclose the empirical evidence of senescence in the different cell populations of the liver, with particular focus on potential markers. By doing so, we would like to provide a comprehensive view of senescence and its consequences in the context of liver homeostasis, disease, and regeneration (see ►Table 1 and ►Fig. 2).

Cholangiocytes and Cellular Senescence

The first description of cellular senescence in bile ducts comes from Lunz and collaborators, who identified senescent cholangiocytes in early stages of chronic liver allograft rejection (CR) and obstructive cholangiopathies.⁹⁵ CR in liver transplant recipients is characterized by cytological alterations in cholangiocytes and ductopenia (disappearance and oblitative arteriopathy of bile ducts) resulting in progressive jaundice, allograft dysfunction, and ultimately a second transplant. Lunz et al demonstrated that ductopenia is preceded by a period of cellular senescence (characterized by p21-positive cholangiocytes that presented shortened telomeres).⁹⁵ Sasaki and colleagues described similar findings⁹⁶ and Brain et al demonstrated a positive correlation between the number of senescent cholangiocytes and an increasing grade of acute rejection, biliary anastomotic obstruction, and stricturing.⁹⁷

Furthermore, Lunz et al show that cyclosporine treatment (an immunosuppressant used at liver transplantation) induces senescence in a TGFβ-dependent manner, potentially aggravating the progression of the cholangiocellular damage rather than contributing to a successful engraftment.⁹⁵ Interestingly, Demirci and colleagues showed increased TGFβ1 in macrophages localizing in close proximity to the portal tracts of liver allografts with CR,⁹⁸ potentially suggesting that communications between macrophages and cholangiocytes might result in TGFβ1-dependent induction of senescence in the later population.

Senescence is present in the cholangiocytes of pediatric patients with varying etiologies,^{99,100} which might indicate pathological development not associated with aging. In disorders that affect cholangiocytes such as biliary atresia,^{99,100} senescence can be observed in hepatocytes. Conversely, in conditions that primarily affect hepatocytes (e.g., tyrosinemia and fulminant hepatitis⁹⁹), senescence can be

observed in both hepatocytes and cholangiocytes, suggesting transference of senescence between liver populations although potential effects of these mechanisms have to be clarified.

Senescence has also been shown to play a critical role in the development of primary sclerosing cholangitis (PSC), an autoinflammatory disorder characterized by fibrosis, stricturing, and obliteration of bile ducts.¹⁰¹ Cholangiocytes in PSC show multiple senescent markers such as SAβGal, p21, p16, γH2A.X, p27, and DCR2.^{96,102,103}

PSC's cholangiocytes are characterized by N-Ras-induced senescence,¹⁰² reduction of Bmi1 (a repressor of p16),¹⁰⁴ and a complex SASP (i.e., IL1α, IL6, IL8, TGFβ chemokine ligand 1, CCL2, and PAI-1 secretion).^{102,103} Components of the SASP has been shown to aggravate the progression of the disease by increasing fibrosis, activating immune surveillance (attracting macrophages both in vitro¹⁰⁵ and in vivo¹⁰³), and inducing secondary senescence in the hepatic parenchyma by means of N-Ras activation¹⁰² and TGFβ-dependent mechanisms.¹⁰³ Moreover, pharmacological abrogation of these pathways inhibited senescence, highlighting the potential for new therapeutic interventions toward PSC treatment in the context of cellular senescence.^{102,103}

Suggested mechanisms of senescence in PSC include regulation by the transcription factor ETS1 and p300 which promotes cholangiocyte resistance to apoptosis by inducing BCL-XL expression.^{106,107} In fact, pharmacological inhibition of BCL-XL with the small-molecule inhibitor A1331852 selectively kills senescent cholangiocytes in the Mdr2 (Abcb4) –/– murine model of PSC,¹⁰⁸ reducing liver fibrosis by means of a PDGF-mediated apoptotic priming of mesenchymal cells.¹⁰⁶

The absence of intestinal microbiota exacerbates biliary senescence in the same PSC murine model and ursodeoxycholic acid (an antioxidant metabolite produced by commensal microbiota¹⁰⁹) is able to abrogate senescence in vitro¹¹⁰ providing an interesting link between microbiota, senescence, and the progression of biliary disease.

Another potential mechanism is derived from the knock-out of secretin receptor (SR, which plays a crucial role in the regulation of biliary damage and fibrosis) in a PSC murine model, effectively reducing the levels of senescence and associated ductular reaction and fibrosis.¹¹¹

In a similar fashion, senescence is present in the bile ducts in primary biliary cholangitis (PBC), an autoimmune condition characterized by granulomatous destruction of bile ducts slowly progressing to fibrosis, cirrhosis, and liver failure.¹¹²

PBC cholangiocytes exhibit senescence markers such as SAβGal, p16, p21, significantly shorter telomeres, and γH2A.X foci indicative of DNA damage.^{96,113–115} Interestingly, p21 expression in some cholangiocytes appears to be independent of p53, suggesting the presence of secondary paracrine senescence mechanisms. This cholangiocyte's SASP might be able to modulate the microenvironment by upregulating the expression of various chemokines such as CCL2 and CX3CL1^{116,117}, promoting the infiltration of inflammatory CX3CR1+ CD3-positive T cells¹¹⁷ and F4/80+ macrophages.¹⁰³ Furthermore,

Table 1 Markers of senescence in the liver, organized per cell type

Effect	Markers	References
Cholangiocytes		
Biliary atresia		
Associated with damage	SaβGal, p53, p21, p16	99,100
Transplantation, obstructive cholangiopathies, acute rejection		
Senescence correlates with damage extension	Lipofuscin accumulation, short telomeres, p21, p16, γH2A.X	95–97
Primary biliary cirrhosis		
Destruction of biliary architecture, immune surveillance activation, lack of regeneration, potential trigger, exacerbation of liver disease	SaβGal, p21, p16, p27, pRb, short telomeres, γH2A.X, DCR2, 53BP1, Bmi1 decrease, IL1α, IL3, IL6, PAI1, TGFβ, CX3CL1, CCL2	96,102–104
Primary sclerosing cholangitis		
Destruction of biliary architecture, immune surveillance activation, lack of regeneration, potential trigger, exacerbation of liver disease	SaβGal, p53, p21, p16, p27, pRb, DCR2, 53BP1, short telomeres, γH2A.X, LC3, cathepsin D, Lamp1	96,103,113–115
Cholangiocarcinoma		
Potential tumor progression through SASP	p16, EZH2 overexpression	196
Hepatocytes		
Acute liver disease: paracetamol intoxication		
Lack of regeneration, immune surveillance activation, secondary senescence	SaβGal, p53, p21, 53BP1, DCR2, γH2A.X	131,132
Nonalcoholic fatty liver disease (NAFLD)		
Senescence correlates with severity and predicts progression of NAFLD, fibrogenesis	SaβGal, p53, p21, p16, p38, pRb, SMP30, 4-HNE, γH2A.X, TAF, SADS, shorter telomeres, acetylation of histones H3/H4, decreased trimethylation of histone H3 at the p21 promoter, decrease of <i>Mcm2cyclin A</i> and PH3, increased CDK4, SNP-related variants of CDKN1a, methylation of SLC7A11, karyomegaly, nucleoplasmic bridges, nuclear buds, micronuclei	82,135,146,151,153–164
Alcoholic liver disease		
Fibrogenesis, adverse outcome,	p21, PAI-1, EGR1, miR-34a,	135,144,145

Table 1 (Continued)

Effect	Markers	References
Viral hepatitis (HBV, HCV)		
HCV: fibrogenesis HBV: protective role by limiting viral replication, antifibrotic	SaβGal, p53, p21, p16, pRb, ΔNp63, Sirt1, GAS2, increase nuclear size, short telomeres	39,134,141–143,146,149
Tyrosinemia		
Effect upon regenerative nodules	SaβGal, p53, p21, p16	99
Genetic hemochromatosis		
Correlation with iron loading and fibrosis stage	p21	148
Hepatocellular carcinoma (HCC)		
Limits HCC development, immune surveillance activation/promotion of tumor development	p53, p21, p16, pRb, ROC1, Sirt1, PANDA,	13,92,93,193–197
Hepatic stellate cells (HSCs)		
Liver fibrosis		
HSC senescence limits fibrosis	SaβGal, p53, p21, p16, pRb, IL22, CCN1, α6β1, PPARC, IGF1 Substance-P/NK-1R,	19,20,181 176–180
Lymphocytes and macrophages		
Lymphocytes (CD3 +, CD4 +, CD8 +) in chronic viral hepatitis, cirrhosis and liver transplantation, NK		
Fibrogenesis, immune exhaustion, viral persistence NK (antifibrotic effect)	SaβGal, p21, p27, p16, p-HP1γ, γH2A.X, telomere length, CD57, PD-1, IL2, ΔNp63-miR-181a-Sirt1 NKG2D	149,182–185 186
Macrophages		
Secondary paracrine-senescence, lack of regenerative response, M1 shift	SaβGal, p53, p21, 53BP1, DCR2, γH2A.X, IFN-γ, IL6	132,188
LSECs		
Pseudocapillarization, microcirculatory dysfunction, perivascular fibrosis	p16, IL1, IL6	190,191

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; SASP, senescence-associated secretory phenotype.

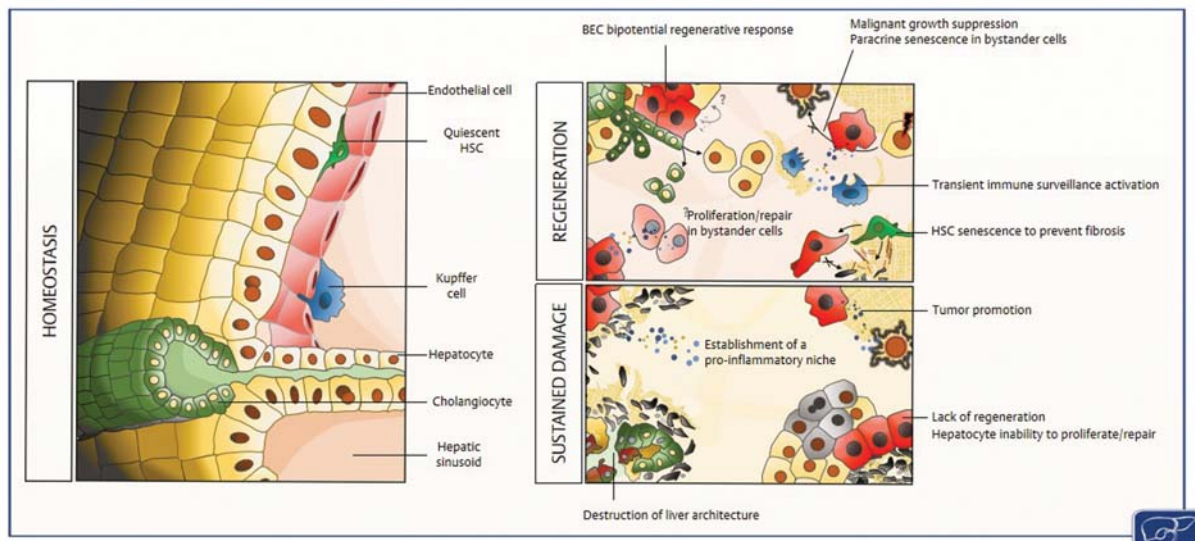


Fig. 2 Effects of cellular senescence in the liver. Left image depicts a normal liver and its populations. There are no known effects of cellular senescence during homeostasis or liver development. Right upper quadrant: beneficial effects of senescence might be associated with an antifibrotic and proregenerative response. Clockwise: senescent cells might induce BEC-bipotential response during liver disease to replace injured cells and promote regeneration and repair.¹⁹⁸ Damaged cells are able to engage senescent programs to avoid unwanted proliferation and tumor progression. Moreover, they are able to induce senescence in neighboring cells to expand the senescent fingerprint and to alert of the damaging agent.^{7,42} Senescent cells elicit a transient immune response that eliminates the damaged cells, promoting regeneration.^{15,30} Senescence in activated HSC prevents fibrogenesis, avoiding further scarring and cirrhosis progression.¹⁹ SASP might also be responsible of inducing proliferation of neighboring cells to promote regeneration and repair. The lower image represents some of the known detrimental effects of senescence at the molecular level. Clockwise, SASP establishes a proinflammatory niche that exacerbates injury.^{11,30,90} Senescent cells promote tumorigenesis.^{30,46} Expansion of the senescent fingerprint to the surrounding cells reduces the regenerative capacity of the tissue.¹⁰⁵ Senescence alters the microenvironment and promotes destruction of the liver architecture, as seen in the bile ducts of PBC and PSC patients.¹⁰⁵ BEC, biliary epithelial cell; HSC, hepatic stellate cell; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SASP, senescence-associated secretory phenotype.

monocytes/macrophages, neutrophils, and plasma cells expressing markers of autophagy such as LC3 and PDC-E2 (pyruvate-dehydrogenase complex-E2, the immunodominant autoantigen of PBC) are found in close proximity to the damaged bile ducts in PBC.^{118,119}

These findings highlighted a potential connection in PBC for senescence and autophagy (critical for the rapid protein remodeling required for the transition from proliferation to senescence¹²⁰). Indeed, PBC cholangiocytes show markers of autophagy including p62 aggregation (p62 sequestosome-1),¹²¹ LC3, LAMP-1, and cathepsin D.¹²² In fact, LC3 colocalizes with the mitochondrial proteins PDC-E2 and CCO,¹¹⁸ and inhibition of autophagy by means of 3-methyladenine significantly suppressed cellular senescence in cultured cells reducing the levels of CCL2 and CX3CL1.¹²²

Together these data suggest a role for senescence in the modulation of cholangiocellular damage and indicate that targeting senescence can potentially improve the conditions and the secondary symptoms associated with the progression of biliary disease. However, the mechanisms underlying these phenomena and their contribution to the onset/development of biliary disease remain unclear. Given that the K19Cre^{ERT2} Mdm2^{-/-} murine model (able to induce senescence exclusively in cholangiocytes) recapitulates numerous characteristics of PBC/PSC,¹⁰³ we suggest that senescence might be an inducer rather than a consequence of

biliary disease. However, the mechanisms leading to this first generation of senescence are yet not clarified.

Oxidative stress (a known inducer of cellular senescence)^{123,124} is characteristic of biliary pathology.^{124,125} The alterations in vascular supply associated with some of these conditions are an important source of oxidative stress¹²⁶ and immune infiltrates contribute to biliary damage through the production of ROS and nitric oxide.⁹⁶ In fact, multiple *in vitro* experiments demonstrated that oxidative stress consistently induces senescence in cholangiocytes.^{96,97,102} However, multiple other mechanisms including DNA damage or serum deprivation are equally capable of inducing senescence in cholangiocytes, highlighting that induction is multifactorial and likely impacts various biological processes.^{96,97}

Telomere shortening has also been described as an inducer of senescence in biliary disease, with short telomeres being characteristic of PBC cholangiocytes.¹¹⁴ However, given that in this study PBC and control liver samples had a similar age ratio, senescence in this context is unlikely to be related to aging, but rather could be a direct consequence of DNA damage.

Autophagy and immunological damage have also been proposed as inducers and/or contributors of cholangiocyte senescence.^{118,121} The SASP is certainly able to induce further damage as well as senescence in bystander cholangiocytes and other liver populations,^{102,103} suggesting that once that senescence is established it might exert further detrimental effects via a positive feedback loop.

Finally, the potential contribution of other insults (i.e., toxic bile accumulation during flow impairment, immune response, portal inflammation, and ECM deposition) to cholangiocellular senescence is worth noting. In summary, there is a complex picture for senescence in biliary pathology that requires further investigation.

Hepatocytes and Cellular Senescence

The hepatocyte changes with age.¹²⁷ Hepatocytes decline in volume¹²⁸ and the nucleus changes morphology while increasing the incidence of polyploidy.^{127,129} Although hepatocytes accumulate markers of senescence in later stages of life,¹²⁹ they are not particularly susceptible to the implementation of senescence in homeostasis¹³⁰ (probably reflecting their high regenerative and restorative capacities). However, senescent hepatocytes are common in a variety of pathologies, indicating that this might be a relevant mechanism in the context of liver disease.

Hepatocyte senescence is strongly induced in the setting of severe acute liver injury.^{131,132} Hepatocytes in paracetamol poisoning are characterized by the presence of SA β Gal, p21, and γ H2A.X, and senescence is able to spread between adjacent cells in a feedback loop dependent on macrophages' TGF β 1 secretion.¹³² This mechanism might initially help to contain the pericentral necrosis seen in paracetamol-derived injury, as the senescent cells seem to surround the damaged areas. However, it actively contributes to the lack of regeneration seen in acute liver injury,¹³² highlighting the potential of targeting senescence and its effectors to restore the regenerative response of the liver.

Similarly, senescent hepatocytes are characteristic of chronic liver disease, a condition caused by sustained liver damage, accumulation of scarring tissue (fibrosis), and eventual progression to cirrhosis manifesting as a deterioration of liver function. In this case, the hepatocyte's telomere length inversely correlates with increasing levels of cirrhosis^{39,133–135} and mutations in telomerase genes TERT and TERC are risk factors for the development of cirrhosis.^{136,137} In fact, murine models in which senescence is induced in hepatocytes through a telomerase knockout show increased fibrosis, accelerated onset of cirrhosis, and reduced survival.^{138,139} Moreover, mice that exhibit a deficient senescent response (e.g., p21 knockout) display reduced CCl₄-induced fibrosis¹⁴⁰ indicating the ability of senescent cells to regulate their microenvironment through the control of ECM deposition and production of profibrotic factors.

Etiologies where presence of senescent hepatocytes have been reported include viral hepatitis B and C virus (HBV and HCV),^{39,141–143} alcoholic liver disease (ALD),^{135,144,145} non-alcoholic fatty liver disease (NAFLD^{146,147}), and genetic hemochromatosis.¹⁴⁸

HBV and HCV exhibit large cellular changes in hepatocytes identified as senescent lesions (with accumulation of p21, SA β Gal, and SAHF with me-H3K9¹³⁴).

In HBV, hepatocytes are found in a p21-mediated G1 arrest, showing decreased markers of late stages of cell-cycle

progression, a decrease of telomere length, and an increase of nuclear size, all indicative of a strong senescent response.¹⁴³ Interestingly, the core antigen of HBV (HBcAg) can only be observed in actively proliferating hepatocytes with intact telomere length. Moreover, p21 is associated with limitation of fibrosis and cell-cycle entry (assessed by *Mcm2* expression) and correlates with increased levels of fibrosis and viral replication.¹⁴³ These results appear to confer senescence a protective role as HBV is confined in young, proliferative hepatocytes, potentially explaining the low viral load in cirrhotic patients with a large number of senescent hepatocytes.¹⁴⁶

Senescence is characteristic of hepatocytes in HCV (with elevated expression of p21, upregulation of *Trp53* and *CDKN2A*,¹⁴¹ SA β Gal accumulation,³⁹ presence of 8-OHdG [a marker of oxidative DNA damage],¹⁴² and a decrease of telomere length,^{39,142} among others). In this context, Sekoguchi et al proposed that senescence may be a consequence of an HCV-dependent hyperproliferative response that induces telomere shortening and increases fibrosis.¹⁴² However, Wandrer et al proposed that immune CD3+ T cells, rather than hepatocytes, are the predominant senescent population in HCV during fibrosis progression.¹⁴⁹ This finding opens new avenues for the selective targeting of different liver populations by inducing host senescence to avoid viral replication or inhibiting immune senescence to contain the infection.

In ALD, the presence of senescent p21-positive hepatocytes is closely related to fibrosis development and an adverse outcome.^{144,150} In fact, 5 weeks of ethanol feeding in mice leads to SA β Gal accumulation and upregulation of senescence initiators (PAI-1 and EGR1) in hepatocytes.¹⁴⁵ Interestingly, silencing of either PAI-1 or EGR1 reduces senescence in human-cultured hepatocytes while decreasing levels of profibrotic markers,¹⁴⁵ suggesting a direct link between the onset of senescence and ALD progression. Similarly, inhibition of miR-34a (which is found increased in clinical samples of ALD¹⁴⁴) decreases overall hepatocyte senescence in ethanol-fed mice while ameliorating fibrosis.¹⁴⁴ Altogether these results suggest a potential role for cellular senescence as a causal event of an effector of ALD progression.

NAFLD and its histological phenotype nonalcoholic steatohepatitis (NASH) encompass a spectrum of conditions, of a broad range of etiologies, characterized by the accumulation of hepatic steatosis in patients who do not consume harmful amounts of alcohol.

NAFLD hepatocytes are characterized by the presence of multiple senescence markers including p53,¹⁵¹ SA β Gal, p21, p16, p38, 4-HNE, γ H2A.X, presence of TAF and SADS, as well as karyomegaly as assessed by morphometric analysis of DAPI.^{82,135,152} As with other chronic liver diseases, hepatocyte senescence correlates with severity of NAFLD.^{82,135,153} Hepatocyte in NAFLD have shorter telomeres,^{135,154,155} increased levels of CDK4,¹⁵⁶ and a significant decrease of *Mcm2*, cyclin A, and PH3, all indicative of lack of cell-cycle progression beyond the G1/S phase.¹³³ Single-nucleotide polymorphism-related variants of *CDKN1A* (p21),¹⁵⁷ acetylation of histones H3 and H4, and decreased trimethylation of

histone H3 at the p21 promoter¹⁵² directly correlate with NAFLD progression.

Further markers of senescence, such as genomic instability (assessed by nucleoplasmic bridges, nuclear buds, and micronuclei)¹⁵⁸ and DNA damage,¹⁵⁹ are also directly related to NAFLD progression. Furthermore, DNA methylation appears to be characteristic of NAFLD,¹⁶⁰ with upregulation of genes associated with lipid metabolism and downregulation of genes related to proliferation and transcriptional regulation,¹⁶¹ methylation of SLC7A11 indicative of reduced steatotic risk,¹⁶² and hypomethylated genes characterizing advanced stages of NAFLD¹⁶³ and changes in nuclear size.^{82,164}

Several senescence-related mechanisms have been causally related to NAFLD onset/progression. For example, as the CDK4-C/EBP α -p300 axis is a critical regulator of NAFLD, pharmacological inhibition of CDK4 results in cellular senescence ablation and reversion of age-dependent steatosis.¹⁵⁶ The reduction of hepatic senescent protein SMP30 (regucalcin, an antioxidant protein that plays an important role in intracellular Ca²⁺ homeostasis and oxidative stress¹⁶⁵) has been associated with NAFLD progression and fibrosis in a stage-dependent manner.¹⁶⁶ Consistently, SMP30 knockout mouse develops characteristics of fatty liver disease,^{167–169} suggesting a direct role for SMP30 deregulation in the development of NAFLD.

Finally, conditional induction of senescence in hepatocytes (using the Alb-Xpg $-/-$ mice that accumulates DDR markers and accelerates karyomegaly in hepatocytes¹⁷⁰) impairs the capacity of mitochondria to oxidize fatty acids, thus accumulating fat in the hepatic parenchyma.⁸² Interestingly, elimination of senescent cells using suicide gene-mediated ablation (INK-ATTAC mice), a combination of senolytics (dasatinib + quercetin⁸²), or caloric restriction¹⁷¹ protects or even reverses hepatic lipid accumulation,⁸² suggesting new therapeutic approaches based on targeting senescence.

As with cholangiocytes' senescence, consistent and marked oxidative injury appears to play a crucial role in the establishment of hepatocellular senescence.

Oxidative stress is a common element of chronic liver disease, contributing to the onset and progression of inflammation and fibrosis.¹⁷² For example, patients with NAFLD show a high level of oxidative stress^{173,174} that contribute to fat accumulation, telomere shortening, and DNA damage in hepatocytes.¹⁴⁶ Senescence also contributes to fat accumulation when mitochondria lose their ability to metabolize fatty acids efficiently.⁸² Therefore, either as a cause or a consequence of the pathology, senescence appears to generate a continuous feedback loop that negatively impacts the hepatocyte and its mitochondrial function. Another example: HCV core protein is able to directly induce oxidative injury and mitochondrial damage.¹⁷⁵ In a similar fashion, telomere attrition and telomerase mutations appear to be predominant mechanisms for senescence induction in hepatocytes^{136,137} although telomere damage is closely associated with oxidative stress.⁶¹

The hepatocyte's role in the metabolism of endo and xenobiotics (including toxins such as alcohol) results in a

constant wave of insults that may compromise their homeostatic balance toward apoptosis, regeneration, or senescence. However, given the complexity and variety of conditions that affect the hepatocytes, the mechanisms that lead to the establishment of primary senescence in this cell type need further clarification.

Other Liver Populations and Cellular Senescence

The role of the hepatic stellate cells (HSCs) in liver disease is intimately related to fibrogenesis, as activated HSCs secrete an excess of ECM components such as collagens and MMP that alter the architecture of the liver and compromise the function of its cells. HSC senescence reduces ECM deposition and increases the level of ECM-degrading enzymes to limit and reverse fibrogenesis.¹⁹ Conversely, a deficient senescent response results in exacerbated fibrosis as HSCs continue to proliferate and deposit ECM.¹⁹

Multiple inducers of senescence have been described in activated HSCs including an increased sensitivity to DNA damage in comparison to quiescent HSCs.¹⁷⁶ For example, upregulation of IL22 promotes HSC senescence and inhibits the expression of α SMA.¹⁷⁷ Indeed, mice overexpressing IL22 have reduced levels of fibrosis.¹⁷⁷

Accumulation of matricellular protein CCN1 in hepatocytes of human cirrhotic livers induces senescence in HSCs and portal fibroblasts through ROS accumulation and an excessive production of integrin α 6 β 1.²⁰ In fact, HSC senescence was required for CCN1 to reverse hepatic fibrosis, and although CCN1 is not essential for liver function or its development, CCN1 deletion in hepatocytes results in exacerbated fibrosis concomitant with a deficient senescent response.²⁰ IGF1 is also able to induce HSC senescence and limit fibrosis in a p53-dependent manner.¹⁷⁸ Indeed, treatment with recombinant IGF1 limits fibrosis by promoting HSCs' senescence via PPAR γ /p53 activation.¹⁷⁹ Further mechanisms of senescence activation in HSCs involve substance-P (SP)/NK-1R axis.¹⁸⁰ Consistent with other studies linking HSC senescence and fibrosis, mice treated with an NK-1R inhibitor display enhanced HSCs' senescence and a reduction in fibrogenesis.¹⁸⁰

In contrast, Yoshimoto et al reported the opposite effects with deoxycholic acid (DCA), which caused DNA damage in HSC and was shown to provoke a SASP phenotype, promoting fibrosis.¹⁸¹ Without resolution, chronic exposure to HSC-SASP promotes fibrosis and may leave patients with NASH susceptible to HCC development. In addition, HSCs appear to have longer telomeres in comparison with hepatocytes in chronic liver disease,^{133,146} potentially indicating that HSCs are less prone to establish a telomere-dependent senescent response, although the biological implications of this finding require further clarification.

To summarize, multiple studies appear to indicate that senescence in HSCs is a beneficial mechanism to avoid excessive fibrosis, although more details are required to fully understand the mechanisms that lead to this phenotype and how to manipulate them to alleviate this burden.

Other liver populations are susceptible to the implementation of cellular senescence, with variable results.

Senescence in lymphocytes has been described in chronic viral hepatitis (CD4⁺,¹⁸² CD3⁺,^{149,183,184} and CD8⁺¹⁸⁵ T cells), cirrhosis,¹³⁶ and liver transplant recipients.¹⁸⁶ This phenotype has been associated with increased fibrogenesis, immune exhaustion, and viral persistence.¹⁸³

NK cell receptor (NKG2D) has also been identified as a crucial factor for the regulation of immune surveillance and protection against liver fibrosis by recognizing MICA and ULBP2 in senescent-activated HSCs.¹⁸⁷

Liver-resident macrophages also participate in the senescent phenotype during paracetamol overdose by spreading senescence to bystander cells via the TGFβ1 ligand.¹³² Senescent HSCs also release factors such as IFN-γ and IL6 that induce an M1 shift in liver-resident macrophages, promoting immune surveillance.¹⁸⁸ Furthermore, given the association of autophagy and senescence¹⁸⁹ and the recent description of “macroph-aging” in old mice, where senescence spreads to immune cells aggravating the pathology,³⁰ we predict that the number of studies relating macrophages and senescence will increase in the coming years.

Finally, aged liver sinusoidal endothelial cells (LSECs) express high levels of p16,¹⁹⁰ IL1, and IL6,¹⁹¹ concomitant to microcirculatory dysfunction and pseudocapillarization.¹⁹¹ LSECs isolated from p19^{ARF}−/− mice are able to escape senescence in vitro without evidencing signs of tumorigenesis¹⁹² and elimination of p16+ LSECs disrupts the blood–tissue barriers inducing perivascular fibrosis associated with health deterioration,¹⁹⁰ evidencing the importance of senescence in the maintenance of homeostasis in this cell type.

Cellular Senescence and Liver Carcinogenesis

Senescence has long been considered an antitumoral mechanism.¹³ In the liver, examples include the establishment of senescence in premalignant hepatocytes to limit cancer development,⁹² SASP-dependent recruitment of immune cells to remove senescent hepatocytes and prevent malignant transformation,^{93,193} and induction of senescence by means of ROC1 ablation to suppress further growth of malignant hepatocytes.¹⁹⁴ Moreover, a defective senescent response is associated with the onset and progression of several liver tumors.^{93,195}

However, it is now clear that senescence may also trigger liver carcinogenesis. Senescence has been associated with the pathophysiology of cholangiocarcinoma by promoting tumor development.¹⁹⁶ Furthermore, it has also been associated with hepatocellular carcinoma (HCC) development,³⁹ where a “senescence-bypass” (product of senescence’s effectors inactivation) leads to DNA damage and chromosomal instability that characterize the progression toward malignancy.¹⁹⁷ Other mechanisms of HCC development include enterohepatic circulation of DCA (a secondary bile acid produced by gut bacteria). This metabolite promotes a SASP phenotype in HSC, facilitating the development of HCC.¹⁸¹

Although these results demonstrate the importance of senescence in liver carcinogenesis, more studies are required

to understand and manipulate senescence to prevent and/or treat liver cancer.

Cellular Senescence and Mechanisms of Liver Regeneration

The liver is an organ with exceptional regenerative properties. During homeostasis, turnover of hepatocytes is modest with < 0.5% proliferating at any given time.¹⁹⁸ However, the liver is capable of completely restoring its mass from the remaining tissue after 75% resection, showing a remarkable ability to enter the cell cycle to restore liver mass.¹⁹⁹

Hepatocyte death followed by the activation of proliferative and inflammatory pathways is traditionally viewed as the main regenerative mechanism in the liver.¹⁹⁹ Liver regeneration is a complex process that can dramatically differ depending on the type of injury, but commonly involves a multicellular response, restoration of the innate-immunity landscape, and revascularization of damaged areas. Within this complex process, senescence appears to play an important role in the mechanisms that determine successful or failed regeneration.

Incidence of liver disease (as well as poorer outcomes) increases with age while the regenerative capacity of the organ decreases.¹²⁷ Accumulation of senescent hepatocytes with age decreases their regenerative rate^{129,200} and, as a consequence, complete restoration of liver mass after partial hepatectomy (PH) is delayed.^{201–203}

In PH (a well-established model of liver regeneration) there is a careful coordination between cell proliferation and cell death that defines the compensatory growth of the organ.¹⁹⁹ In this setting, several studies have identified senescent markers p21 and p27 in hepatocytes,^{204–206} with p21 reaching maximum levels in a p53-independent fashion at 48 hours after peak DNA synthesis.^{200,205} The hepatocytes of p21-deficient mice progress faster into the DNA synthesis phase, while p21 overexpression impairs hepatocyte proliferation after PH.^{205,207} Furthermore, secondary hepatocyte senescence (in a model in which primary senescence is induced in cholangiocytes) failed to regenerate after 48 hours of PH, suggesting that senescence controls an effective liver mass recovery.¹⁰³

In fact, hepatocytes in adult mouse livers subjected to PH presented high levels of p21 expression and SASP factors but lacked other senescent markers such as p16 or SAβGal.²⁰⁰ Although p21 alone may not represent a standard senescent phenotype, genetic deletion of p21 partially rescued liver regeneration. Furthermore, treatment with the senolytic drug ABT737 decreased p21 and SASP expression while improving liver regeneration, highlighting its potential for regenerative-based treatments.²⁰⁰

Overall, these results suggest that senescence is a protective mechanism at 48 hours after PH (when the rate of DNA damage peaks alongside the increase in DNA synthesis). However, if the balance between proliferation and senescence in the remaining hepatocytes tilts toward senescence, the regenerative ability of the liver will be compromised, although senotherapeutics showed promising results as potential enhancers of regeneration.²⁰⁰

Senescence might also be a control mechanism to prevent excessive growth beyond the point of repair, where the liver function is already restored. Similar mechanisms of developmental senescence have been described to pattern embryo structures, again in a p21-dependent and p53-independent manner.^{27,28}

The SASP might be partially responsible for this response, as it has been shown to induce cellular plasticity,¹⁸ activate stem cells,¹⁸ and provide signals critical for cellular reprogramming.¹⁷ A murine model of hepatocyte senescence subjected to further dietary damage shows a strong activation of biliary epithelial cells (BECs) with bipotential capacity to become cholangiocytes or hepatocytes and restore liver mass and function.^{208,209} This suggests that in a situation where senescence in the hepatic parenchyma renders this population unable to proliferate and repair the injury, the liver still retains alternative regenerative pathways based on stem cell-related mechanisms to prevent further damage, regenerate the damaged areas, and restore liver function. Further studies will define in the future if the SASP of these senescent hepatocytes includes factors that facilitate this regenerative response (► **Fig. 2**).

Targeting Cellular Senescence: the Future of Therapeutics in Liver Disease?

Cellular senescence is a complex phenotype and its consequences for liver homeostasis, disease, and regeneration are still under study. In the liver, senescence is a highly antagonistic pleiotropic response, dependent on a spatial/temporal context. As a protective element, short-term senescence is associated with antitumoral, hepatoprotective, antifibrotic, and proregenerative mechanisms.^{11,15} However, it has also been linked to impaired regeneration, carcinogenesis, and progression of inflammation.^{7,11,15,31}

Cellular senescence underlies most types of chronic liver disease, where the persistence of inflammation and the alteration of the fine balance between regeneration and destruction might lead to the implementation of senescence. However, murine models have shown that senescence per se is able to recapitulate the onset key events of several liver conditions, suggesting a causative role for this phenomenon.¹⁰³

Either as a triggering factor or a consequence of the pathology, senescence has proven to be a relevant factor in liver disease. As so, several groups propose to use senescence markers to help establish diagnosis and severity of chronic conditions (such as p21 in CR,⁹⁵ chitotriosidase in HCV,¹⁴⁹ or SMP30 in NAFLD¹⁶⁶). However, the necessity of finding several factors and lack of superficial noninvasive markers complicate the use of senescence as a diagnostic tool.

Studies looking at the effects of preventing, reducing, or inducing senescence in the context of liver disease are currently under way. For example, manipulation of host senescence could block hepatitis virus infection,¹⁴² while inhibiting immune senescence could prevent the spread of hepatitis viruses.¹⁴⁹ Induction of senescence in HSCs could reduce fibrosis,¹⁹ while blocking hepatocellular senescence might be advantageous to reverse cirrhosis^{133,138} and treat NAFLD.¹³⁷ Blocking of SASP factors could prevent the detri-

mental effects of cholangiocyte senescence in PBC¹⁰³ and promote liver regeneration in the setting of acute liver damage.¹³² Furthermore, controlling senescence-related pathways, such as autophagy or apoptosis, might be another strategy to manipulate this phenotype therapeutically.⁴⁶

In line with these findings, several groups have reported the use of novel agents able to induce senescence (curcumin,¹⁷⁹ tetramethylpyrazine,²¹⁰ retinoic acid,²¹¹ or dihydroartemisinin²¹² in HSCs) or eliminate senescent cells by means of senolytics (such as A-1331852,¹⁰⁶ dasatinib + quercetin⁸²) to restrain the damage and induce liver regeneration. Selective elimination of senescent cells may be key to the success of senolytics as demonstrated recently in a mouse model of HCC in which therapy-induced senescent cancer cells were specifically targeted with the mTOR inhibitor AZD8055, and the survival time was almost doubled.²¹³

Partial blockage of SASP factors has also proven to be a useful strategy to manipulate senescence effects.^{103,132} Other strategies aimed at modulating senescence in other tissues might be useful for treating liver conditions, such as metformin administration,²¹⁴ caloric restriction,²¹⁵ or use of nanocarriers.²¹⁶

Modulation of senescence by means of senolytics, senomorphics, or SASP-blocking agents could also help us to manipulate the intrinsic regenerative abilities of the liver. By doing so we might be able to promote an endogenous BEC-dependent reparative response and improve the engraftment and proliferation of transplanted cells used in cell therapy.²⁰⁸

The aging population, combined with the increasing incidence of liver disease, has sparked a new wave of interest in senescence related to hepatology. Because of the inherent complexities of the senescence process, it will be crucial to understand the mechanisms and consequences of senescence in liver disease to develop effective senescence-targeted therapies.

Moreover, study of emerging fields such as epigenetics^{217,218} that could influence the senescence response in the liver in association with other pathologies (such as obesity²¹⁹), “macrophage-aging” and its implications for immune surveillance^{30,220} and its associated “inflammaging”^{221,222} (characterized by the upregulation of the inflammatory response that occurs with advancing age), will immensely contribute to the understanding of liver disease and regeneration.

Main Concepts and Learning Points

- Cellular senescence plays a relevant role in the occurrence and development of liver disease.
- We provide a comprehensive list of senescence markers and related mechanisms in liver homeostasis and disease.
- We discuss the implications of cellular senescence for liver regeneration.
- Manipulation of cellular senescence as a potential therapeutic intervention.

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Conflict of Interest

The authors declare they have no conflict of interest.

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